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REVIEW

LARGE DNA SEPARATION USING FIELD ALTERNATION AGAR GEL ELECTROPHORESIS

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CONTENTS

LIST OF ABBREVIATIONS

CHEF	Contour-clamped homogeneous electric field
FIGE	Field inversion gel electrophoresis
OFAGE	Orthogonal field alternation gel electrophoresis
PFGE	Pulsed field-gradient gel electrophoresis
RGE	Rotating gel electrophoresis

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TAFE	Transverse alternating field electrophoresis
kb	Kilobase pairs of nucleic acid
Mb	Megabase pairs of nucleic acid
CpG	Cytosine guanine

1 INTRODUCTION

Size, charge and conformational differences have been the basis of molecular separations across the spectrum of scientific enquiry In most electrophoresis applications, a single pair of electrodes are used to generate a uniform field in a single direction. In these instances separation is achieved by the charge of the molecule and the sieving capacity of the gel matrix. The ability to separate molecules based on size, however, is usually restricted within upper and lower molecular mass ranges, which result from limitations of the system in question. These limitations are particularly evident in macromolecular DNA electrophoresis [1,2]

Conventional agarose gel electrophoresis separates native DNA molecules, within the range 1–40 kilobase pairs (kb), on the basis of molecular mass Above the upper molecular mass threshold size of 30–40 kb, DNA molecules exhibit size-independent mobilities and co-migrate in an agarose gel matrix [1,2] The shape adopted by DNA in gels and the reasons for the co-migration phenomenon are not fully understood However, in the same manner that separation of DNA molecules is dependent on the relationships of molecule size, the pore size of the matrix and the strength of the electric field, so the co-migration of DNA must be a result of these same relationships [1–3]

A practical look at some of the pertinent facts relating to the size of DNA molecules and of the agarose gel matrix is very helpful in understanding agarose gel electrophoresis and the co-migration phenomenon The average pore size of 1–1 5% concentration agarose gels is approximately 90–120 nm [3–5] The length of lambda phage (48 5 kb) on the other hand is approximately 16 μm [6] and its radius as an unextended random coil, as it exists in solution, is approximately 500 nm [3,5–7] Given these observations and that the persistence length of DNA is 50–60 nm [3,5,7] it would seem that in order for the molecules to enter the gel they must orient longitudinally with respect to the gel pores [3,7] This orientation occurs under the influence of the electric field applied across the gel [3,7]. As the pores and pore sizes are not regular throughout the agarose gel matrix, the forward motion of the large molecule is thought to be serpentine in nature as it migrates through pores of adequate size With the beginning of serpentine motion, which has been termed 'reptation' [8,9], the capacity of the gel matrix to sieve the molecules is lost because the charge on the DNA and the friction exerted on the moving molecules are both proportionate to size Consequently, all large molecules in a constant electric field and a homogeneous gel matrix are subjected to the counter-productive

forces of forward motion and friction. Therefore, any movement of large molecules is dependent on gel pore size and field strength and is no longer a function of molecular mass [3].

The molecular mass limitations imposed by the co-migration phenomenon of DNA have been extended by the use of low-agarose-concentration gels This approach has enabled separation of DNA up to 750 kb [1]. The gels, 0.1–0.5% agarose, usually require very low voltages, at times as low as 0 02 V cm^{-1}, and very long running times [1] All of these factors contribute to diffuse DNA bands and, of course, the gels are very difficult to manipulate. Recent innovations in agarose gel electrophoresis have substantially altered the way in which DNA separations are achieved and have dramatically expanded the upper molecular mass boundaries permitting separation of very large DNA molecules and intact chromosomes of many organisms These innovations are also adding to our understanding of how DNA migrates through a gel matrix

2 PULSED FIELD-GRADIENT GEL ELECTROPHORESIS

In 1983, Schwartz et al. [10] addressed the problem of separating large DNA molecules based on data from viscoelastic techniques for DNA molecular mass determinations [2,8] and the understanding of the co-migration phenomenon of DNA [1,2,8,9] They theorised that, by forcing large molecules to periodically change direction and taking into account the strongly size-dependent relaxation time of large DNA [8], it would be possible to open new dimensions of size-dependent DNA separation In order to achieve these directional changes, a complex electrophoresis tank electrode geometry was designed in conjunction with an electrical switching unit [10,11]. This enabled the periodic application across the gel of two different electric fields at right angles to each other in the horizontal plane The electrode array geometry was manipulated, as were the pulse times of the alternating fields, until large DNA was resolved on the basis of molecular mass [11] While all units with complex electrode arrays operating with alternating fields can be described as pulsed field-gradient gel electrophoresis (PFGE) units [10–30], this term will be reserved in this article for the array depicted in Fig. 1.

PFGE uses a single non-homogeneous field in alternation with a homogeneous electric field to achieve separation [10–14] The electrode array of the homogeneous field consists of equal numbers of vertical diode isolated platinum wires at the cathode and anode, while the non-homogeneous array consists of a series of electrodes for the cathode and a single-wire anode The diode isolation of each electrode prevents the electrodes from carrying current or distorting the field while the opposite array is operating The electrode geometry has a marked effect on the fields generated and as a consequence on both the electrophoretic pattern of the DNA separation and the molecular mass separation range In addition, separations are markedly influenced by the pulse

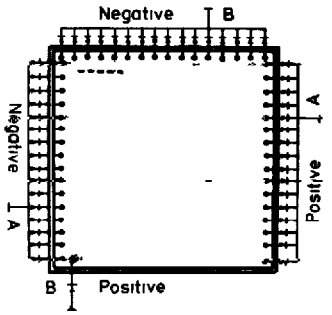

Fig 1 Schematic diagram of PFGE as described by Schwartz and co-workers [10,11] This electrode geometry has been widely adapted and modified [12–18] The gel sits submerged in buffer within the electrode format The samples are loaded into gel slots (solid bars) and the current applied alternately to the A and B electrode circuits giving a pulsed movement of East–West then North–South Each electrode wire in the array is protected by diodes (arrows and bars) against current leakage or conduction when the opposite array is in operation The electric fields generated (dotted lines) when the electrode array is engaged are either homogeneous (A) or a non-homogeneous gradient (B) The net effect of the two fields can be judged by the intersection points of both fields assuming equal pulse time or voltage It is obvious that the effect is not the same on all samples, consequently sample migration is not straight, which makes lane-to-lane comparisons difficult

times and the field strengths employed as well as temperature and the agarose concentration of the gel [15]

Separation is normally achieved using a longer pulse time for the non-homogeneous field than for the homogeneous field or alternatively by using differing voltages with the lower voltage being applied across the homogeneous field This latter procedure, while more common, necessitates the use of two electrophoresis power supply units [12–14]. The DNA separation patterns that result with PFGE are quite spectacular in appearance (Fig. 2) The DNA paths have almost a three-dimensional appearance with the path appearing to leave the wells, travel to the uppermost surface of the agarose, then down to the lowest surface only to rise up and go down again, all the time sweeping down the length of the gel in arcs. Despite these tortuous migration patterns, the molecular mass separations and the band widths achieved are quite acceptable [11–18] The lane-to-lane comparisons, however, are very poor. An additional problem is that, to compensate for the migration path of the DNA in the PFGE system, the sample wells are located in an extreme edge of the gel (Fig 1) This prevents the samples running off the agarose gel during the electrophoresis but means that only a few samples can be run on a gel particularly if any realistic lane-to-lane comparisons are to be made.

Considering the nature by which the DNA separation is achieved in PFGE it would seem unlikely that the bands should resolve as anything but smears, however, another force is at play in the field alternation separation In a non-

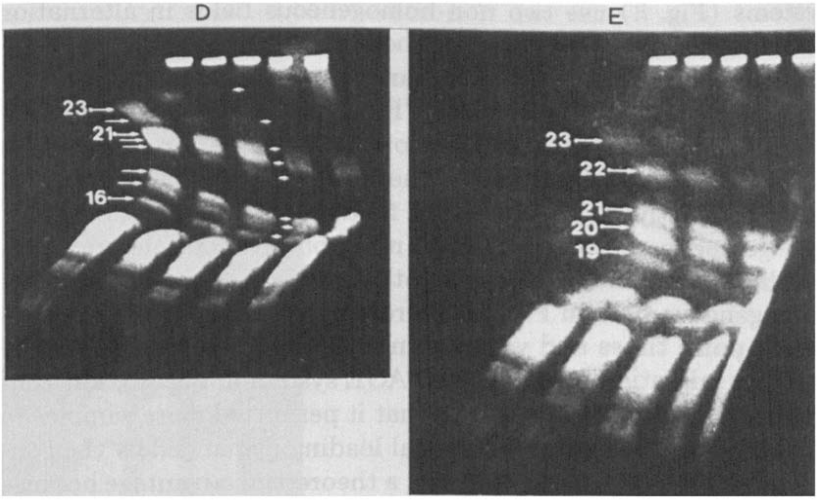

Fig 2 PFGE separations of the chromosomes of *Leishmania major* V121 This separation was performed using a modified PFGE electrode geometry which was the mirror image of that shown in Fig 1 [12] Naturally, the gel pattern is also the mirror image of that expected from the array in Fig 1 Using 1% agarose instead of 1 5% agarose with other conditions remaining constant, the authors were able to resolve chromosomes 19-23 (E) which had run as a compressed group in (D) The parameters for these PFGE separations were 200-s pulse time with 12 5 V cm^{-1} in the A array and 5 V cm^{-1} in the B array over 48 h The temperature was kept constant at 16°C (Reproduced with kind permission of the authors, Spithill and Samaras [12])

homogeneous field, the field strength progressively weakens A consequence of this is that the DNA at the front of the band experiences a weaker current than at the rear of the band, resulting in a sharpening and focusing effect on the DNA bands. Thus the separations achieved were possibly much better than a theoretical appraisal of the electrode geometry and field alternations would have suggested

3 ORTHOGONAL FIELD ALTERNATION GEL ELECTROPHORESIS

In addition to the PFGE electrode arrangement described, Schwartz and co-workers [10,11] also described a PFGE with two non-homogeneous fields While this was, in actual fact, the first pulsed field-gradient unit described [10] it was subsequently modified [11] This concept was still further modified by Carle and Olson [20] who termed their array orthogonal field alternation gel electrophoresis (OFAGE) OFAGE employs continuous electrodes rather than the vertical diode isolated electrodes used by Schwartz and co-workers [10,11], but the theoretical basis of separation, the field geometry and the electrophoretic patterns are similar Consequently, for simplicity and clarity, both arrays will be described in this publication under the single title of OFAGE

OFAGE systems (Fig. 3) use two non-homogeneous fields in alternation giving the net forward direction at a diagonal between the arrays [11,15,20,21] The angle of the alternating electric fields change throughout the tank and the gel but are between 100 and 150° While PFGE and OFAGE are effective, better resolution and separation as well as more linear migration patterns are achieved with OFAGE [11,20,22]. This is the result of a number of features working in concert. Firstly, a more balanced field alternation due to the balanced electrode geometry Secondly, the two non-homogeneous electric fields result in band sharpening and focusing in both directions compared with the single non-homogeneous field in PFGE Thirdly, the electrode geometry operates with equal pulse times and voltages in both arrays and consequently produces straighter migration paths. The OFAGE system in Fig. 3b, was considered to have an additional advantage in that it permitted more samples to be loaded than was possible with the diagonal loading demanded by the configuration in Fig 3a. This, however, was only a theoretical advantage because there were still many problems with lane-to-lane comparisons in the 'hourglass' shaped OFAGE separations [16,17,20,21,31,32] Consequently, only a small number of samples could be loaded in the middle wells of the gel if the migration paths were to be compared with any accuracy (Fig. 4). OFAGE systems of the type depicted in Fig. 3a [11] are marketed by Pharmacia LKB

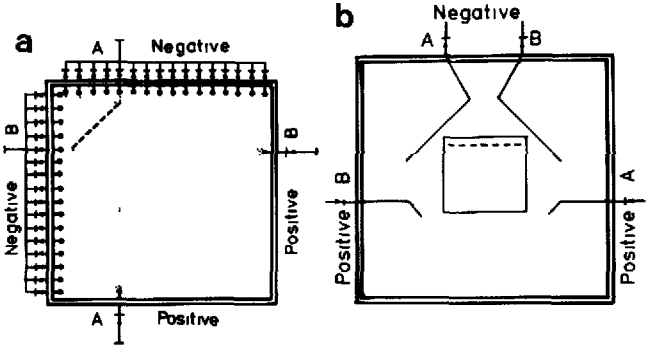

Fig 3 Two types of gel electrophoresis electrode formats (a) Electrode geometry described by Schwartz and co-workers [11,12], the basis of the Pulsaphor apparatus (Pharmacia LKB), (b) electrode geometry described by Carle and Olson [20,21] The field geometry in both systems is very similar and consequently both have been referred to in this article as OFAGE This terminology is not used by others to describe (a) but for the sake of clarity it has been adopted here The oblique angle and the two non-homogeneous fields give better separation of large DNA molecules than observed with PFGE The net effect of the alternating field gradients is seen in the intersection of the dotted lines which represent the electric fields and thus the diagonal migration path of the DNA is potentially straighter than for PFGE (compare Figs 2 and 4) There is also a more consistent effect on all the samples (solid bars) than occurs for PFGE which results in better lane-to-lane comparisons The migration pattern, although nearly parallel at the beginning, usually fans out in the low molecular mass range of the gel which can make sample comparisons difficult (Reproduced from Dawkins [33])

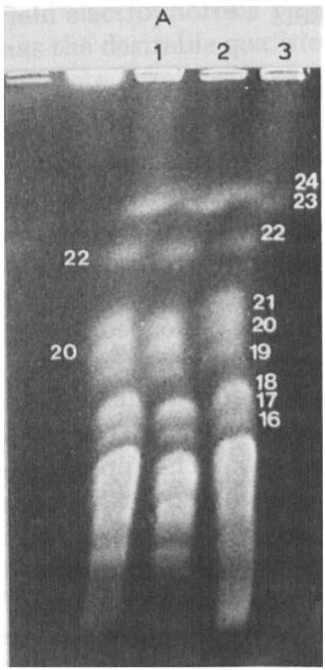

Fig 4 OFAGE separation of the chromosomes from three cloned lines of *Leishmania major* The electrode array used in this study was very similar to that depicted in Fig 3a The straighter migration of samples loaded in the middle sample wells of the gel improves lane-to-lane comparisons The separation parameters were 1 5% agarose, with a 320-s pulse time and 5 V cm^{-1} for a total electrophoresis period of 67 h The temperature was constant at 4°C (This figure was kindly donated from unpublished results of Drs Nick Samaras and Terry Spithill, Walter and Eliza Hall Institute for Medical Research, Melbourne, Australia)

(Uppsala, Sweden) under the trade name of Pulsaphor and have recently been demonstrated to give separation of molecules up to 10 Mb [34]

A further PFGE modification which should be discussed here was described by McPeek et al [19] Their modifications gave very similar electrophoretic patterns to OFAGE and on occasions gave separations that demonstrated straight-lane migration The modification described, however, involved a very elaborate switching system and an electrode array consisting of four non-homogeneous electric fields which were applied in clockwise rotation The results were good, but the results from later and more simplistic electrophoresis developments overshadowed their elaborate modification As is true of rapidly developing areas, many worthwhile designs and techniques are surpassed even before the publication goes to press The ideas, however, are to be admired and the additional information provided increases our understanding of how large DNA migration and separation occurs

4 TRANSVERSE ALTERNATING FIELD ELECTROPHORESIS

Gardiner et al [24] produced a vertical gel electrophoretic system that essentially adopted the OFAGE configuration of Carle and Olson [20,21] The design and capability of this unit appear to have been grossly underrated, and despite the fact that it was the first field alternation electrophoresis system to achieve straight-lane migration of large DNA fragments they received very few citations The pre-occupation with linear migration patterns is not one of aesthetic appearance but is an issue of a fundamental and functional nature as it permits better lane-to-lane comparisons, more samples to be loaded per gel and easier interpretation of the results These considerations are very important, particularly when the gels are to be used in hybridisation studies

The unit, as depicted in Fig 5, had two alternating fields at 120° to each other but the angle has subsequently been optimised at 115° The application of the alternating fields results in the DNA migrating back and forth across the thickness of the gel and then progressively down the length of the gel The gel, usually 5 mm thick and of a 1% agarose concentration, is of a minigel format which is supported on each edge by vertical perspex slots on the side of the tank The resilience of the gel is required to hold it firm and upright in the tank. The electrode arrangement produces a homogeneous field effect throughout the gel giving straight-lane migration patterns over the entire area of the gel The fact that the gel has to be poured on a template then loaded into the gel tank does present some handling difficulties, however, these are a very minor consideration The electronic switching unit allows great flexibility in running parameters.

To date there are only a small number of papers using transverse alternating

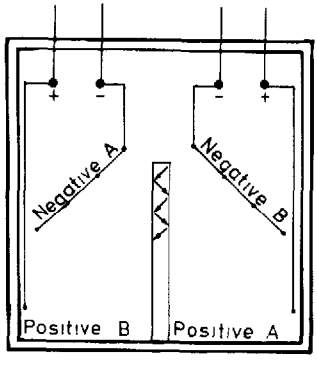

Fig 5 Diagrammatic representation of the TAFE system (ref 24 and Geneline, Beckman Instruments) which is in essence a vertical OFAGE The alternating field angles of 115–120° result in the molecules switching back and forth in the thickness of the gel The electrode arrangement produces a homogeneous field within the gel which results in straight-lane migration of samples throughout the gel The Geneline unit is marketed by Beckman Instruments

field electrophoresis (TAFE), although more can be expected as this system has the desirable qualities of straight-lane migration, is simple to use and can separate chromosomes up to 10 megabase pairs (Mb) (Geneline brochure) TAFE systems are marketed by Beckman Instruments (Palo Alto, CA, U S A.) under the name Geneline.

5 FIELD INVERSION GEL ELECTROPHORESIS

A further innovation, the result of experimentation with less complex electrode geometries [25], occurred at the same time as the TAFE modification [24]. It was demonstrated that periodic inversion of a homogeneous electric field resulted in the effective separation of large-molecular-mass DNA molecules The technique of field inversion gel electrophoresis (FIGE) uses conventional agarose gel electrophoresis apparatus in which the homogeneous field is periodically inverted. Forward motion of the molecules results as a function of the set ratio of the field inversions, for instance a ratio of 3 1 gives 3 units forward to 1 reverse (Fig. 6a). Increasing the time period of the field inversions in a continuous ramp over the running time of the gel, while maintaining a set forward to reverse ratio, results in a molecular mass-dependent separation of the DNA The separations achieved with FIGE produce straight-lane migration which gives accurate lane-to-lane comparisons but the bands tend to be more diffuse when compared with the other systems, particularly in the higher-molecular-mass ranges. A modification of FIGE which gave additional control over the electrophoretic parameters was achieved using vertical gel electrophoresis units [26] This enabled control of the voltage actually passing through gel and overcame the heating problems experienced by the other field alternation systems. The vertical FIGE utilised low voltage which only passed through the gel and consequently permitted very simple but effective temperature control of the system [26,33].

FIGE made large DNA separation available to all laboratories with standard electrophoresis equipment as the only requirement was for a switching unit capable of altering the variables of pulse time and ratio Carle et al [25] used a programmed personal computer with an external switching unit to alter their electrophoretic parameters, while this laboratory used an integrated microprocessor controlled switching unit [26,33] There are now many commercial manufacturers of FIGE switching units which permit precise control over the variables of pulse time, gradient, ratio and the total time period of the electrophoresis run. These include the PC 750 pulse controller (Hoeffer Scientific Instruments, San Francisco, CA, U.S A), Pulsewave 760 (Bio-Rad Labs , Richmond, CA, U S A.), Sidewinder (Kontes Life Science Products, Vineland, NJ, U S.A) and the PP1-100 (MJ Research, Cambridge, MA, U S.A)

Despite the inability of FIGE to exceed 2-Mb separations and the more diffuse bands in the upper molecular mass range, this system still remains one of

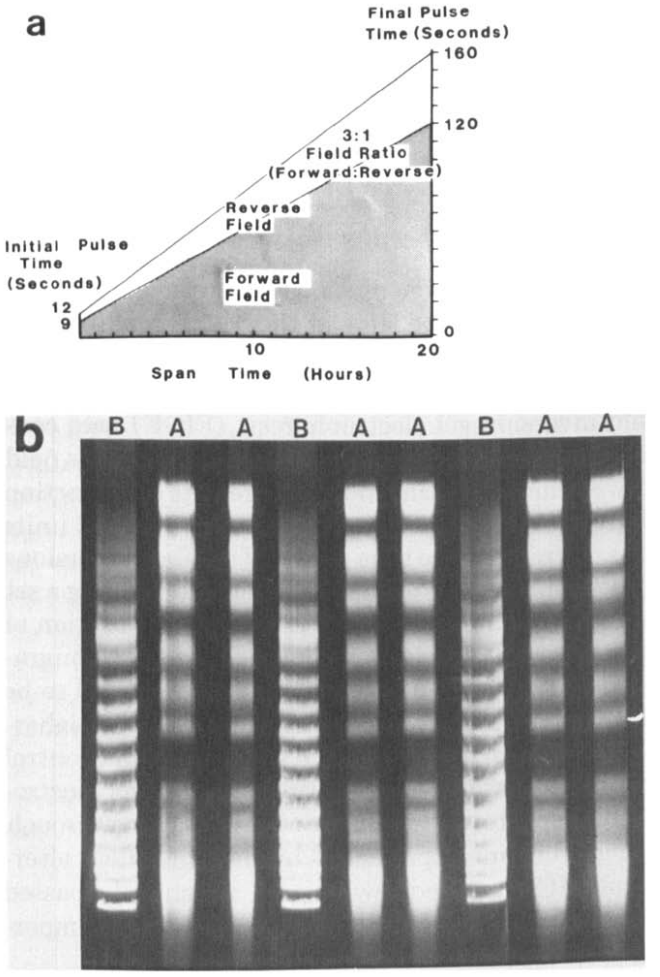

Fig 6 (a) Running parameters used in FIGE The pulse ramp is usually linear between the beginning pulse time and the final pulse time The field inversion ratio of forward to reverse is usually 3 1 and is set throughout the run period The steepness of the ramp is dependent on the parameters of run time and beginning and final pulse times As the ramp increases so the forward pulse time also increases and larger molecules are able to migrate into the gel matrix The ramp is very important for large DNA separation [25,26,33] (b) FIGE separation of *Saccharomyces cerevisiae* chromosomes (A) and lambda oligomers (B) The electrophoresis parameters were 12 s beginning pulse time, 90 s final pulse time with a 3 1 forward/reverse ratio over a total running time of 20 h The voltage was constant 3 75 V cm^{-1} and the temperature 14°C (Reproduced from Dawkins et al [26])

the most easily accessible large DNA electrophoresis systems The upper molecular mass limitation should only be considered a restriction when large chromosomes need to be resolved, but in the case of large restriction fragment analysis or smaller chromosome bands (less than 1 5 Mb) FIGE is a valuable

technique and has many advantages over OFAGE [33]. FIGE is also the only technique that gives separation of *Giardia intestinalis* chromosomes (ref 35 and Upcroft, personal communication) The reasons are uncertain, but it is considered that the tertiary structure or molecules associated with the native DNA influence the separation of *Giardia* chromosomes which seems to complement the mechanism by which FIGE separates DNA.

6 ROTATING GEL ELECTROPHORESIS

FIGE had demonstrated that field gradients were not essential for large DNA separation Based on theoretical and practical extrapolation from OFAGE and FIGE an apparatus was developed with a homogeneous, uniform electric field and a rotating gel [27,28]. This rotating gel electrophoresis (RGE) unit, affectionately referred to as the 'Waltzer' because of the gel rotation, operated in a conventional homogeneous electric field which enabled many of the separation parameters to be tested [28] A circular gel was required to maintain the homogeneity of the field at every point in the gel. The degree of rotation while capable of being set to any angle was found to be optimal for most separation parameters when angles between 110 and 120° were used The electric field is only interrupted for the few seconds it takes the gel to rotate to its second position. Other than this, the separation is achieved with a constant voltage and a homogeneous field. A schematic representation of the Waltzer [27,28] is shown in Fig 7.

Of all the apparatus dealt with in this manuscript perhaps the simplest and most effective is the Waltzer The electrophoresis is undertaken with a con-

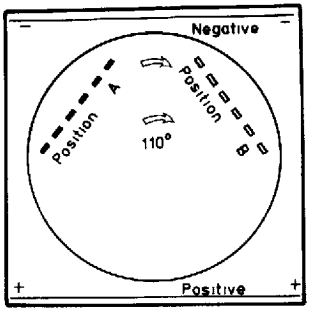

Fig 7 Diagrammatic representation of the RGE apparatus of Anand [27] and Southern et al [28] The gel is subjected to a homogeneous electric field, however, the circular gel rotation between positions A and B produces a field alternation on the samples The optimal angle of the gel rotation is between 110 and 120° The rotation of the gel prompted people to refer to this system as the 'Waltzer' The electric field is only interrupted for the few seconds it takes the gel to rotate to its new position This unit is not yet commercially available, however, an RGE which uses electrode rotation rather than gel rotation to achieve a similar effect is being marketed as Rotaphor (Biometra)

ventional electrode array, the optimal angle for gel rotation is determined and fixed and the gel is subjected to a constant pulse time. If desired, a second pulse time can be programmed to begin after a certain period during the electrophoretic run time to facilitate better separation over a wider range of DNA As with TAFE, Waltzer will separate chromosomes up to 10 Mb in size (Fig. 8) and gives straight-lane migration for each sample loaded. It would seem that the most complex aspect of Waltzer is pouring gels on a circular template

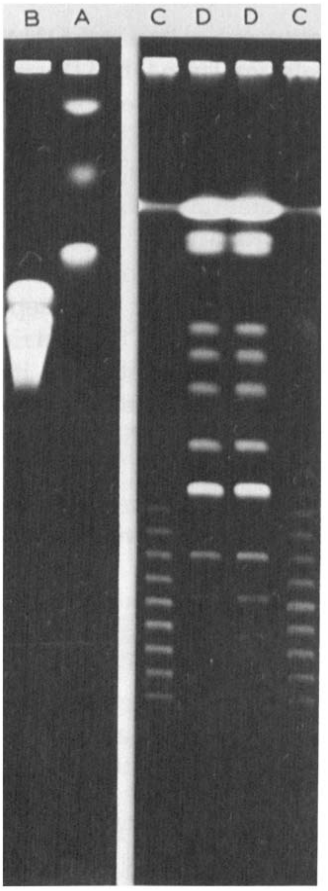

Fig 8 RGE (Waltzer) separation in the first gel showing (A) the three chromosomes of *Schizosaccharomyces pombe* of 3, 6 and 9 Mb and (B) a compression of the larger chromosomes of *Saccharomyces cerevisiae* not showing any separation The smaller chromosomes would have run off the gel under the separation conditions used here of 0 7% agarose, 1 2 V cm^{-1} with 60-min switching times over a period of seven days The temperature was 5°C The second gel shows separation of (C) lambda oligomers and (D) the chromosomes of *Saccharomyces cerevisiae* The separation conditions for this gel were 1 5% agarose, 6 V cm^{-1} with 60-s switching times over a period of 33 h The temperature was 20°C (Reproduced with kind permission of Dr Rakesh Anand [27])

RGE, as with nearly every system discussed in this paper, has also been modified The Rotaphor apparatus uses concave electrodes, mounted on the electrophoresis tank lid which move around a stationary rectangular gel, to achieve a homogeneous alternating electric field The principle is of course the same as that of Waltzer and the operation of the field alternation angle is the mirror image of that determined by Anand [27] and Southern et al [28]. The RGE systems have the additional advantage of being able to alter the rotation or move the electrodes through 90° after a separation and then undertake a second field alternation separation, thus producing two-dimensional large DNA separations This may be particularly useful with some protozoal parasites which, very frequently have deletions and rearrangements in some chromosomes. However, while this procedure can be undertaken more easily with RGE it can also be fairly easily achieved with gel manipulation in the other systems

A commercial Waltzer is not yet available, however, the Rotaphor is manufactured by Biometra (Gottingen, F.R.G) and is marketed by a number of companies internationally

7 CONTOUR-CLAMPED HOMOGENEOUS ELECTRIC FIELD ELECTROPHORESIS

At the time RGE was described [27], the development of the contour-clamped homogeneous electric field (CHEF) [29] was in press. CHEF operates from an hexagonal electrode arrangement in which each electrode in the array has a clamped electric potential The first model had four vertical electrodes in each of the six arrays [29]. Subsequent models have four horizontal electrodes in each array [30] The clamped electrode potentials are determined by a series of resistors In this way the voltage of the operating array determines the voltage of the remaining four electrode arrays (Fig 9). The effect of this is a homogeneous electric field at all points within the rectangular gel. The electrode array, while complex, is very efficient and is easily operated. To set the electric field the operator determines the voltage of the operating electrode array, the resistors then act to 'balance' the field through the other electrodes to produce a homogeneous field. The pulse times for each array are the same so that the net effect of the alternating 120° fields is to cause the DNA molecules to migrate down the length of the gel, in much the same manner as RGE with the homogeneous field ensuring straight-lane migration (Fig 10)

As with TAFE and RGE, CHEF separations are straight and effective up to 10 Mb [29,30] In all of these systems the electronic switching units permit the gel run to be divided into two separate switching intervals which gives better separation over a very much wider molecular mass range. CHEF is marketed by Bio-Rad Labs under the trade name CHEF DrII In addition, Pharmacia LKB have released an electrode array for converting the Pulsaphor (OFAGE) apparatus into a CHEF unit While OFAGE have given separation

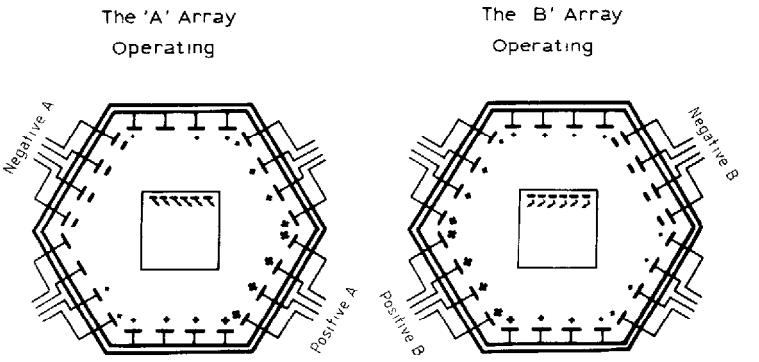

Fig 9 Diagrammatic representation of the CHEF electrode format showing the clamped potentials at each electrode (represented by the size of the positive sign) when the A and B electrodes are in operation The original CHEF [29,30] had vertical electrode wires as does the Pharmacia LKB CHEF conversion electrode The CHEF DrII (Bio-Rad Labs) has horizontal electrodes on the base of the electrophoresis tank The clamped electrode potentials produce a homogeneous electric field throughout the gel and a field alternation angle of 120°

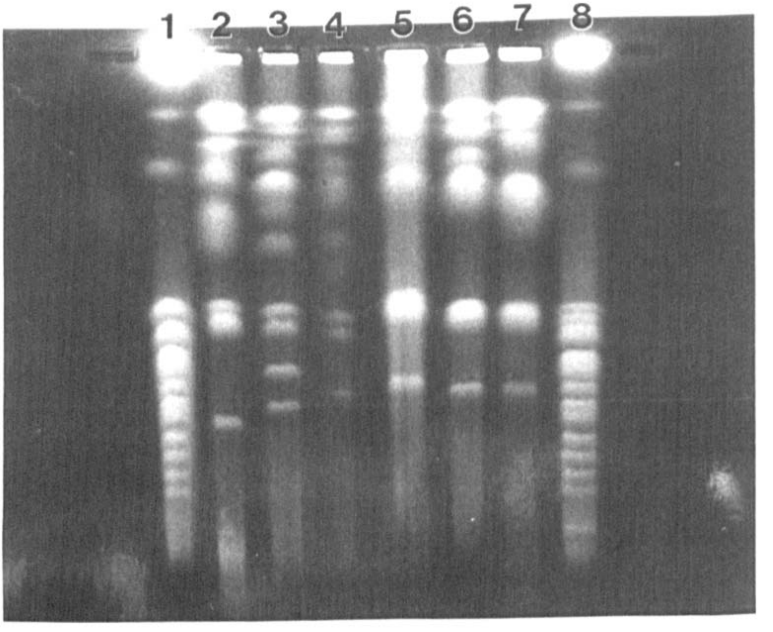

Fig 10 CHEF separation of the smaller *Plasmodium falciparum* chromosomes (lanes 2, 3, 4, 5, 6 and 7) demonstrating chromosomal rearrangements in these organisms Lanes 1 and 8 contain the resolved chromosomes of *Saccharomyces cerevisiae* The conditions were 1% agarose, 120 V with 150-s pulse times over a period of 22 h The temperature was 12°C The CHEF apparatus used in this instance was made from original plans supplied by Chu et al [29] (This figure is reproduced with the kind permission of David Kemp [23])

of DNA molecules up to 10 Mb [34], the CHEF electrode array permits the same size separations with straight-lane migration.

8 COMPARISON OF FIELD ALTERNATION ELECTROPHORESIS SYSTEMS

The potential applications of field alternation electrophoresis are enormous, however, their adoption in research laboratories were hindered for a number of reasons. Initially, the complex electrode geometry of PFGE and OFAGE, the absence of commercial electrophoresis tanks, electronic switching units and the need for buffer recirculation and cooling systems all contributed to a hesitancy by some groups to enter this new field. The frequent requirement of two power supply units to achieve the desired electric fields in the two arrays also contributed to the restricted spread of this technique. The most limiting aspect of PFGE and OFAGE, however, was the small number of samples and the complex migration patterns of the DNA

One of the most important criteria in large DNA separation is straight-lane migration as it is essential for accurate lane-to-lane comparisons and molecular mass estimation The reproducibility and accuracy of the straight-lane migrations produced by TAFE, FIGE, RGE and CHEF are all very comparable. The molecular mass range over which chromosomes can be separated and the sharpness of the resulting bands, however, are much better in TAFE, RGE and CHEF. These three units all use the optimal field alternation angles of 110–120° [24,27–30] and are capable of resolving the sixteen chromosomes of *Saccharomyces cerevisiae* and the three very large chromosomes of *Schizosaccharomyces pombe*, approximately 3, 6 and 9 Mb.

Field alternation gel electrophoresis procedures are usually performed at temperatures ranging from 4 to 16°C, with high voltage and current output to achieve the separations. The normal voltages for most separations are 10 V cm^{-1}, which over the 20–30 cm of the gel boxes amounts to 200–300 V The heat generated by these high voltages has been calculated to be about 60 W [15], which of course has made buffer cooling and recirculation essential to maintain a constant temperature throughout the 20–48 h these gels were run Despite the numerous modifications to the field alternation electrophoresis systems, the problems of joule heating have not been overcome, except in the case of vertical FIGE [26,33] This means that all the units described require efficient heat exchange and, as such, cooling systems are an essential requirement of field alternation electrophoresis

In view of these comments it is not really possible to distinguish between the TAFE, RGE and CHEF systems as each has similar sample capacity, separation ability and price Waltzer does have a very slight advantage of electrode simplicity and particularly clean DNA separation, although the circular gels present a barrier, albeit small, to acceptance. As this unit is not yet commercially available it seems to a large extent academic, however, plans are made

available on request [27,28]. The ease with which the FIGE can be adopted by a laboratory still makes it an attractive technique, particularly where large restriction fragment studies are to be undertaken or where the total chromosomal length of the organism in question is less than 2 Mb.

9 THEORY OF ELECTROPHORETIC SEPARATION OF LARGE DNA

It would seem that there are many theoretical models to describe how very-large-molecular-mass DNA is separated by the techniques described but there are also a number of practical observations about the behaviour of DNA that need to be taken into consideration Consequently, the best picture of what is happening in the agarose gel can probably be derived by considering the theoretical models and the practical observations simultaneously.

Theory demands that very large molecules take a longer time to orient in an electric field than relatively smaller molecules [2,8] and in view of their size and conformation in relation to the gel pore size, longer to enter the gel matrix The field alternation pulse time determines how far the molecules travel, or if indeed they enter the gel pores Once in the gel, the molecules migrate as a flexible rod, travelling in a serpentine motion through the matrix. As the DNA is forced through the pores of a gel, one end of the molecule is thought to lead the movement in the direction parallel to the applied field. Movement of this end is always in a forwards direction and results in the trailing end becoming aligned with the field Thus the molecule winds its way through the gel pores of the agarose matrix along the path opened up by the leading end [3,9] but is in constant alignment with the electric field.

With the application of another pulse from the other electrode array the molecule must re-orient. How this re-orientation occurs, however, is the crucial question In the model put forward by Southern et al. [28] the molecules re-orient from the trailing point of the DNA molecule and then enter a gel pore to migrate in the new field direction; this is schematically represented in Fig. 11. Others have assumed that a major re-orientation occurs between the successive pulses [10,11,15,20,21,30], but have not specified how it occurs Whatever the mechanism, it is apparently the same for all the field alternation systems

It has been demonstrated that in 1% agarose gels, the centre of mass of a single lambda molecule moves approximately 5.8 μm with a 1-s 10 V cm^{-1}pulse [36]. A 3-s pulse would result in the centre of mass of the lambda molecule moving 20 μm, a distance greater than the 16 μm contour length of this molecule [6,36] In actual fact under similar conditions (1 5% agarose and 8 V cm^{-1}) the extended length of the lambda molecule has been shown to be only 5 5 μm, not 16 μm [28] Thus, the extended form during agar gel electrophoresis is less than one third the value proposed by other techniques which are used to calculate contour length [6,36] Consequently in 1 s the lambda mol-

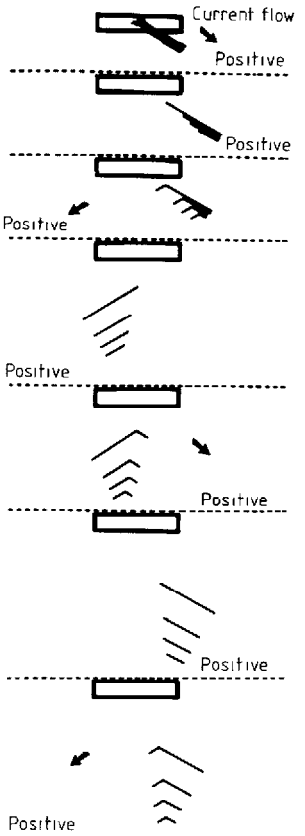

Fig 11 Representation of the theoretical model proposed by Southern et al [28] as the mechanism by which large DNA molecules separate in field alternation electrophoresis The molecules enter the gel and comigrate with the leading point of the DNA molecules moving the same distance Field alternation (gel rotation) causes the molecule to re-align, which it does by moving from what had been the trailing end The turning point is fixed by the gel matrix causing the molecule to ratchet Thus the molecules' movement is determined by the pulse time with larger molecules spending a longer period of each field alternation ratcheting and with smaller molecules spending a longer time migrating down the gel While the molecules are re-orienting, the non-aligned portion of the molecule is subjected to increased friction by the electric field which has the effect of further decelerating these molecules These theoretical mechanisms for DNA separation are consistent with all the field alternation systems, with the single exception of FIGE

ecule moves far beyond the gel pores originally occupied. This demonstrates that while field strength influences molecular conformation the field strengths used in field alternation electrophoresis do not totally extend the molecules A very similar finding has been reported with closed circular supercoiled DNA which have exhibited very much smaller extended lengths than were predicted by molecular mass and contour length (reviewed in ref 15) Nevertheless, the

DNA molecules in the electric fields are to some degree extended, and this extended form is thought to be stabilised by the gel matrix It also gives a clue as to the mechanism of large DNA separation and the observations on the molecular mass separations achieved

Once large molecules are extended within the gel they co-migrate with the leading edges moving together. With alternating fields, the current is stopped and a new field applied at an obtuse angle (110–120°) to the first field These extended molecules must re-orient to align themselves to the new field The theory proposed [28] suggests that the molecule now leads off from what had been its trailing end. This is energy conversing and produces the least friction on the turning molecule The molecules follow the new leading end but to completely align with the new field they must pass the pivotal point, which is the position last occupied by the original trailing edge before the field alternation The larger the molecular mass of the DNA the longer it will spend ratcheting around the pivot point and, therefore, will not be travelling down the gel. Conversely, relatively smaller molecules will spend more time migrating down the gel and less time turning about the pivot point. In addition to this, while the molecules are turning and seeking to re-align with the new field, extra friction is applied to the molecule at each point along its non-aligned length and at the pivot point Naturally this results in a further deceleration of the molecules proportionate to their size. Thus, as has been demonstrated in all systems [11,20,24,25,28] the molecular mass separations are strongly dependent on pulse time and also very much dependent on field strength

In the case of very large molecules the relatively short pulse times would be insufficient to fully extend the molecules Nor would they be thought to begin to migrate, as to do this, the molecule would have to pass the point occupied by the leading edge at the beginning of the pulse time. Thus logic would predict that very large molecules would have trouble entering the gel and would probably not migrate in an alternating field Such is not the case, as very large molecules do enter the gel and migrate a considerable distance considering the low field strength and the relatively short pulse times. These molecules could be migrating with the leading end being turned at a very acute angle and then being dragged back in front of its original path In this case the very large molecules in a relatively small switch time would move due to a zig-zag motion of the alternating field always turning the leading edge. This would account for the small migration distance and the slightly broader band width seen for large molecules More likely, however, is the situation where the molecule bends and folds back on itself thus migrating as a double thickness molecule [15,28]. This shape, while reduced in size experiences, increased friction and has a lower migration rate but is now capable of penetrating the gel further than would be theoretically postulated So migration occurs, but separation using the same relatively short field alternation parameters does not occur

Practical observations tend to confirm the theoretical outline except for the

fact that there appear to be three regions involved in large DNA separation [28,30], the first being very well described by the model and observations of Southern et al [28] in which separation occurs based on a simple extended molecule. The second area would appear to be better described by less extended molecular structures indicating that perhaps a tertiary structure is involved in determining the mobilities of these molecules [15]. A third area is known as the compression zone in which molecules, far above the molecular mass of those being separated, migrate considerable distances into the gel. This could be viewed as a possible extension of the second mechanism where the natural folding of the molecule is even more severe and the migration is accounted for by the doubling over of the molecules as discussed. These observations have been made by a number of authors [15,28,30] and are consistent with the theories for migration

The only apparatus which operates from slightly different principles to the other field alternation systems and which could explain its inability to separate molecules greater than 2 Mb is FIGE It could be viewed that much of the separation achieved with FIGE results when the large coiled or folded molecules begin to orient from one point and this end leads the migration and then stops when the field inverts The molecule then re-orients from another point which begins to move in the opposite direction. As the pulse times increase in the ramp so these partly coiled conformations are able to elongate until the molecule is completely aligned, and consequently can migrate, with the effect that molecular mass separation occurs It would seem that FIGE works from similar principles to those in the other systems In particular, the second region of separation discussed above would seem to fit the observations on FIGE separations, but is more dependent on shuffling molecules apart because of the 180° angle of the field alternations [25,26,33]. The method by which FIGE separates seems to be highly suited to separating the chromosomes from some organisms, such as *Giardia* [35].

At present no theory is totally adequate to explain DNA migration in conventional agarose gels [1,3,4,9] and consequently explanations of what occurs in field alternation electrophoresis is less satisfactory In spite of these deficiencies, a number of complementary theories seem to give a coherent picture of how the DNA is separated and permit at least rudimentary calculations to determine optimal pulse times, running times and field strengths required to achieve a particular molecular mass range separation

10 SAMPLE PREPARATION AND ELECTROPHORESIS CONDITIONS

In preparing this manuscript it was felt that a methods section and some discussion of electrophoresis parameters and conditions may have been helpful The effect of electrophoresis parameters on large DNA separation, however, is the subject of a recent review [15] Commercially available field alter-

nation electrophoresis systems also contain handbooks with very detailed sections on conditions influencing separation The effect of conditions are also detailed in a number of other publications [15,27–30,34] In addition, many papers give clear reproducible methods, and so, to better use the space available, it was decided to simply list relevant methods The methods recommended here are working methods with sufficient information for any experimenter to repeat the procedure A number of the methods cited are used in this laboratory or are known to have been used successfully by other workers In recommending these methods consideration has also been given to the accessability of the published work

Yeast chromosomal markers are usually prepared from known strains of *Saccharomyces cerevisiae* [11,24,27,28,30], however, *Candida albicans* [14,30] has also been used To determine the separation capabilities of electrophoresis units the fission yeast *Schizosaccharomyces pombe* with its three large chromosomes of 3, 6 and 9 Mb has proved useful [29,34] A number of companies are now making *Saccharomyces cerevisiae* for use as molecular mass standards with Beckman Instruments and Bio-Rad Labs. being two international companies marketing these products

Lambda phage oligomers (ladders) are a particularly useful molecular mass marker as each band differs by one lambda molecule (48.5 kb in most cases) Commercially available preparations of lambda virons are not usually suitable for making oligomers because the cohesive ends have often been damaged in the industrial process and consequently only make very small ladders (four molecules) This may be rectified in the future when companies recognise the specialist market, in which case it will probably be possible to purchase lambda oligomers The recommended technique [28,29] produces ladders that extend up to 1 Mb (twenty molecules) and are easily stored Two other suitable techniques for lambda oligomers are referred to in refs 30 and 37

Techniques for the preparation of mammalian cells [24,27,30,38] and parasites such as malaria [13], *Cryptosporidium* [39] and *Leishmania* [12,40] have been documented as have techniques for the preparation of bacteria [22,41] In many instances chromosomes are too large to resolve by any of the instruments described here In this case restriction endonucleases that recognise 6 or 8 base pairs and in particular those that recognise the cytosine guanine (CpG) dinucleotides are used to cleave these chromosomes as they cut the DNA very infrequently We use the technique outlined in ref. 27, however, other techniques are quite similar [22,24,30,32] For a choice of infrequent cutting enzymes see refs. 27 and 42

Running conditions for gels are usually 10 V cm^{-1} with field alternation intervals set according to the molecular mass separations required Separation of large DNA up to 9 Mb is usually performed for a number of days at low temperatures and voltages, 1.5–2 V cm^{-1} [28,34]

Techniques for transfer of large DNA onto nitrocellulose or nylon mem-

branes are the same as the procedures for conventional DNA transfers and hybridisation

There are some drawbacks to large DNA separation of which the principle one is the inability to accurately determine the molecular mass of molecules above 1000 kb. In fact the only way to accurately size the DNA bands in the 1–10 Mb region is to cleave these molecules with restriction enzymes into subfragments which can be sized This form of two-dimensional field alternation electrophoresis has other significant advantages which will be mentioned in the next section One final point on size determination is that quantitative densitometry has been suggested to provide an accurate and linear measurement of DNA up to 2 Mb [15] Unfortunately the requirement for a densitometer makes such a method less appealing than estimating molecular mass with markers such as lambda oligomers and yeast strains with known chromosome sizes

11 APPLICATION AND FUTURE

All the systems discussed in this article have brought significant advances to the field of nucleic acid electrophoresis One simple advantage that has arisen is the ability to prepare intact chromosomal DNA [10]. This is possible because the organisms or cells are embedded and digested in agarose blocks which gives great stability to the DNA and makes its storage and handling very simple In fact, the whole procedure is far simpler than the conventional procedures for genomic DNA preparation. In addition, this technique overcomes many of the problems associated with conventional DNA preparation such as DNA strand shearing and the hazards of working with toxic chemicals Restriction fragment length polymorphism analysis can also be made more accurate by using intact chromosomes rather than genomic DNA. This simple method for routine preparation of chromosomal DNA has universal application

The more functional advantages of large nucleic acid separations are that, in the case of yeast and protozoa, it has been possible to separate entire intact chromosomes [12–32,35,36,40], which permits electrophoretic karyotyping of these species While much of the published work has been directed towards yeast [11,14,19–21,26–30,34] significant work has also been conducted on *Plasmodium* [13,23,31], *Leishmania* [12,40], *Trypanosoma* [17,32], *Giardia* [35], *Cryptosporidium* [39] and *Neurospora*, a filamentous ascomycete (yeast) [43] This ability to separate chromosomes enables cloned sequences to be assigned to their chromosome and simplifies the identification of chromosomal re-arrangements and deletions. In addition, the isolation of individual chromosomes means that the chromosome of interest can be purified and analysed and restriction digestion of these chromosomes is used to give a clearer picture of chromosomal alternations, in effect giving two-dimensional large DNA separations This technique is particularly useful in malaria and trypanosome re-

search where the organisms express deletions and re-arrangements on a number of chromosomes. The ability to separate chromosomes up to 10 Mb is further increasing the scope of field alternation with lower eukaryotes

In the case of the mammalian genome, chromosomes are too large for separation to occur. The smallest human chromosome is estimated to be 50 Mb while that of the mouse is 70 Mb. This necessitates that the DNA be digested in agarose blocks [24,28,38] by restriction enzymes that cut infrequently in the mammalian DNA This technique results in a limited number of fragments which fall within the molecular mass capabilities of the field alternation electrophoresis units, i.e 50 kb to 9 Mb. The enzymes used usually recognise 6–8 base pairs and often require CpG dinucleotides in their recognition sequence. These CpG restriction enzymes actually cleave the DNA less frequently than would be theoretically estimated. Firstly, because the CpG dinucleotide sequence is underrepresented in mammalian genomes and secondly because these sites frequently have methylated cytosine residues which inhibit the cleavage [18] The use of these enzymes have permitted the physical mapping of very large continuous sections of mouse and human genomes [16,18,42,44,45] Physical maps of up to 1.5 Mb can be constructed using restriction enzymes and a single DNA marker. By further using 'linking' libraries [16,23,38,42,44,45] it is possible to electrophoretically map entire regions within mammalian genomes Such techniques are currently being applied to the major histocompatibility complex [16,45], Duchenne muscular dystrophy [38], cystic fibrosis [44] and understanding of the CpG cluster islands of mammalian genomes, known as HFT islands [18,42]. These procedures are also being used to map the genome of bacteria which range in size from 1 to 6 Mb [22,41] In addition, linkage mapping through cosmid clone walking along the large restriction fragments improves the efficiency of genomic mapping and simplifies the identification of repeat sequences [16,23] Large DNA separation also permits the isolation of large polycistronic insertion fragments

Since the region of interest in the genome of any species can be identified by hybridisation analysis it is possible to work on entire genes or gene clusters contained in a large DNA fragment As with yeast and protozoal chromosomes, it is possible to cut the region of interest from a gel and then analyse, cleave or purify the fragment of interest The ability to identify the area of interest in mammalian DNA and remove it to work further on this band reduces the complexity of the enzyme-treated genome by more than 50 times [42] which in itself is a worthwhile and simplifying approach

The field alternation systems have enabled a 500-fold increase in the size of DNA molecules that can be fractionated on agarose gels This technique has entirely altered the genetic analysis of bacteria, yeast and protozoal parasites The application of field alternation electrophoresis to mammalian genetics has, in conjunction with novel molecular biological techniques, so transformed this field that the once impossible gap between classical genetics and molecular

biology has been bridged This means that genetic linkage maps, derived from cytogenetic techniques, are being confirmed, altered and more definitively mapped Such is the confidence in field alternation electrophoresis that scientists now believe it is possible to map entire chromosomes and even entire mammalian genomes [22,27,38,42,44,45] Field alternation affords a new and powerful method for examining the organisation of megabase pair expanses of DNA

As new methods are developed to cope with this quantum leap in nucleic acid separation and new endonucleases are identified which have highly selective cleavage sites, so the enormous power of this technique will be more fully realised

12 SUMMARY

The techniques for large DNA separation have developed from a seminal idea for field alternation which has transformed the field of DNA electrophoresis This single innovation of pulsed field-gradient electrophoresis (PFGE) and the subsequent modifications have made a significant impact on molecular biology, eukaryote genetics, biopolymer research and diagnostic research

The apparatus types used for large DNA separation are depicted and critically compared with relation to molecular mass separation capabilities, straight-lane migration of samples, band sharpness and ease of operation With these criteria in mind PFGE and orthogonal field alternation gel electrophoresis systems had a number of drawbacks, the principle one being the inability of these systems to give straight-lane migration To a large extent this has restricted the widespread use of these systems Field inversion gel electrophoresis produces straight-lane migration but was subject to an upper molecular mass limitation of 2 megabase pairs and tended to produce broader bands in the higher-molecular-mass areas. Transverse alternating field electrophoresis, rotating gel electrophoresis and contour-clamped homogeneous electric field electrophoresis systems where superior to all the other systems They gave straight-lane migration, separation of chromosomes up to 10 megabase pairs, good resolution of bands and were all relatively simple to operate. Very little was found to separate these three electrophoresis systems.

Field alternation electrophoresis has enabled a 500-fold increase in the size of DNA molecules that can be resolved in agar gels Consequently, electrophoretic karyotypes of a number of organisms have been produced, while genome maps, gene locations and sequences of large areas of mammalian genomes are now being undertaken The ability to separate entire chromosomes or large DNA fragments has, in conjunction with novel molecular biology techniques, enabled scientists to work backwards from large purified fragments or entire chromosomes to construct long-range genetic maps The time saving alone

when compared with the old techniques of using very small fragments to construct a picture of the gene or gene complex is commendable

The diagnostic role of large DNA separation and electrophoretic karyotyping is beginning to be explored, while the use of this technique for clinical studies of genetic disorders is well advanced

Very few innovations in nucleic acid separation have had as marked an influence on as many areas as field alternation electrophoresis These techniques have brought mapping of the mammalian genome into the realms of possibility and is contributing in many spheres to the understanding of molecular organisation.

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